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14. ABSTRACT

1. We established an IGFBP5 mutant in the nuclear localization motif and breast cancer cell lines that stably express the mutants as well as wild type form 2. We have constructed fluorescent fusion protein (pDsRed) with both wt IGFBP5 and nuclear localization signal mutant IGFBP5 and evaluated their cellular localization. We also evaluated cellular localization of the untagged forms of these two proteins. We found that mutation of the nuclear localization signal altered the IGFBP5 localization from nucleus to being secreted outside the cells. Further, the secreted form of IGFBP5 is resistant of a cleavage activity. 3. We studied the behavior of the mutant IGFBP5 in two assays: DNA synthesis and cell migration. We found that in contrast to wild-type IGFBP5, which inhibits DNA synthesis and cell migration, mutant IGFBP5 promotes DNA synthesis and cell migration. These results support our hypothesis that localization of IGFBP5 determines its cellular function.

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Table of Contents

Final Report on W81XWH-05-1-0485 (July 15, 2005 – July 14, 2006)

Title: The cellular localization of IGFBP5 determines its oncogenic functions in breast cancer

PI: Wei Zhang, Ph.D., The University of Texas M. D. Anderson Cancer Center

1. Introduction

Background: Metastasis is a major problem in breast cancer, and the molecular mechanism is not well understood. Using cDNA microarray and tissue microarray technologies, other groups as well as our group found that insulin-like growth factor binding protein 5 (*IGFBP5)* expression is associated with a poor prognosis and metastasis in breast cancer patients (Van't Veer *et al* 2002; and Hao et al., 2004). The mechanism by which *IGFBP5* promotes metastasis and hence a poor outcome is unknown. A major difficulty in understanding the function of IGFBP5 is the paradoxical observation that ectopic overexpression of IGFBP5 in breast cancer cell lines resulted in a suppressed proliferation (Butt et al., 2003, and our unpublished observation). A more detailed analysis of IGFBP5 in breast cancer tissues and in transfected breast cancer cell lines showed that IGFBP5 is located in different compartment in the cells. In cancer tissues, IGFBP5 is in the cytoplasm; in the transfected cell IGFBP5 is in the nucleus (also see Schedlich et al., 2000). Thus, we hypothesize that localization of IGFBP5 determines its functional outcome of the host cells.

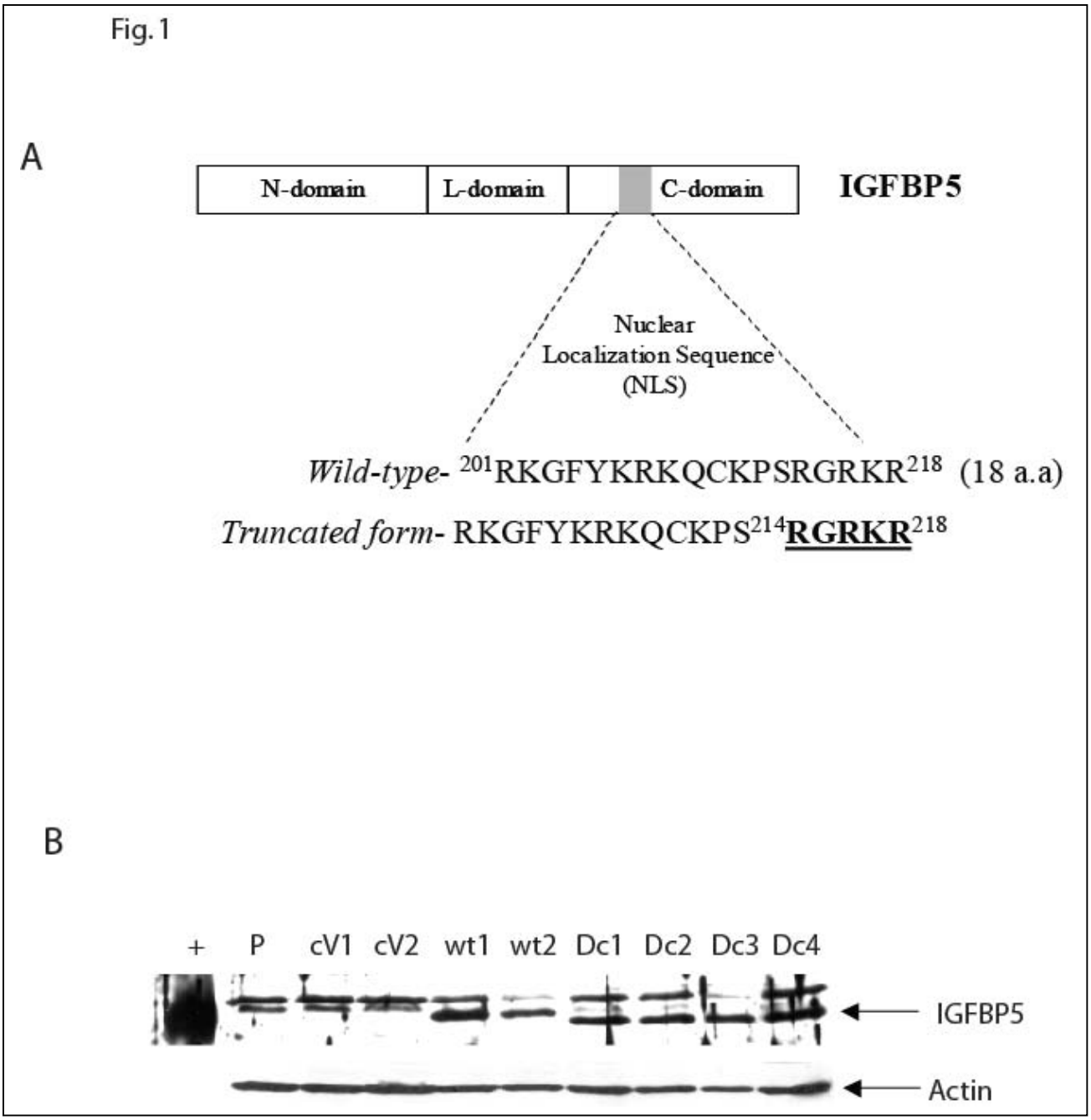
Rationale/Purpose: Genomic studies have identified IGFBP5 as a potential target for therapeutic intervention. This effort suffered a setback by the surprising results from transfection experiments. Our further studies found a potential mechanism that explains the paradox. However, our hypothesis needs to be validated, a critical step toward the realization of IGFBP5 as a therapeutic target. This is the rationale and purpose of this concept grant application.

Objectives: The goal of specific aim 1 is to generate an IGFBP5 mutant in the nuclear localization motif. This mutant will be tagged by red fluorescent protein (RFP). The goal of specific aim 2 is to transfect the new construct into breast cancer cells and confirm its cytoplamic localization. The goal of specific aim 3 is to compare the phenotypes in the transfected cells either by wild-type IGFBP5-RFP or mutant IGFBP5-RGP.

2. Body

1. We established an IGFBP5 mutant in the nuclear localization motif and breast cancer cell lines that stably express the mutants as well as wild type form. The results are shown in figure 1. **A** Schematic diagram of the IGFBP5 cDNA fragments used for generating the IGFBP5 expression constructs. The wild type IGFBP5 contains a nuclear localization sequence (NLS) located at the C-terminal of the protein from amino acid 201 to 218 and indicated by a gray solid square. The full length of IGFBP5 cDNA was generated by RT-PCR using the total RNA from MCF7 breast cancer cell line. The sequence of the primers used is as follows: forward primer: 5'-GCCACCATGGTGTTGCTCACCGCGGTCCTCCTGC-3', and reverse primer: 5'-TCACTCAA-CGTTGCTGCTGTCGAAGGTGTG-3'. A Kozak consensus

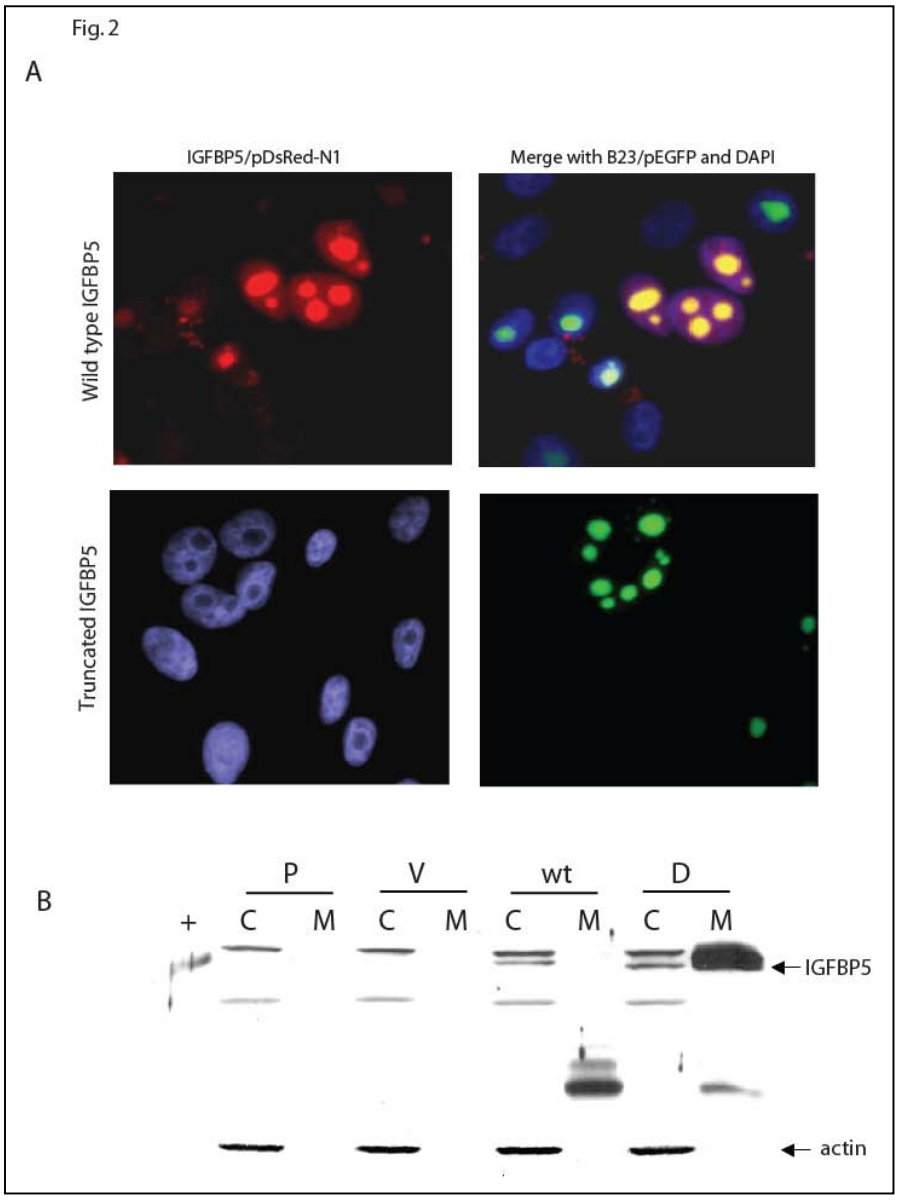
GCCACC is flanked in front of IGFBP5 translation initiation site to increase the translation efficiency. The PCR product was inserted into pCR 2.1TOPO plasmid (Invitrogen) and several clones were chosen for DNA sequencing. The IGFBP5 cDNA with accurate sequence was digested off from the TA-cloning plasmid sequence using EcoRI restriction enzyme and inserted the cDNA into the EcoRI site of the pcDNA3.1(+) expression vector (Invitrogen). And again several clones were sequenced and the clone with right orientation and cDNA sequence of IGFBP5 was chosen to perform the transfection and generate IGFBP5 stable cell lines. The mutant form of IGFBP5 construct was generated by the QuickChange Kit (Stratagene) using the wild type IGFBP5/pcDNA3.1(+) expression plasmid as a template. As shown in Fig. 1A, 5 amino acids in the NLS from 214-218 (black characters) were deleted. **B** Western blot analysis of IGFBP5 expression. MBA-435 breast cancer cells were stably transfected with either wild type or mutant form of IGFBP5 construct and the whole cell lysate from parental MBA-435 cells and different stable lines were subject for western blot analysis (40 μg/lane) using an anti-human IGFBP5 antibody. The blot was re-probed with actin to normalize for protein loading.



Parental and vector transfectant (V) have no detectable IGFBP5. wt: wild type and d: deletion form

IGFBP5.

2. We have constructed fluorescent fusion protein (pDsRed) with both wt IGFBP5 and nuclear localization signal mutant IGFBP5 and evaluated their cellular localization. We also evaluated cellular localization of the untagged forms of these two proteins. We found that mutation of the nuclear localization signal altered the IGFBP5 localization from nucleus to being secreted outside the cells. Further, the secreted form of IGFBP5 is resistant of a cleavage activity. The results are shown in figure 2. **A** Wild type of IGFBP5/pDsRed-N1 or D-IGFBP5/pDsRed-N1 fusion construct was transiently transfected into MBA-435 breast cancer cells and the images were taken by fluorescence microscopy. B23/pEGFP fusion construct was co-transfected to indicate nucleolus structure. The cells were counter stained with DAPI. The wild type IGFBP5 showed strong nuclear localization, especially in the nucleolus: co-localization with B23 protein (yellow color). However, truncated form of IGFBP5 (D-IGFBP5) lost nuclear location capability. **B** Western blot analysis of IGFBP5 expression in both cell lysate and conditioned medium. One million cells as indicated were cultured in 100mm diameter tissue culture dish in the complete medium for 24h followed by 24 more hours incubation in the serum-free medium. Both cell lysate and conditioned medium were collected. The conditioned medium was concentrated three times using YM-10 column (Millipore) and 40 μg of protein and 45 μl of the concentrated conditioned medium were subject for western blot analysis. No IGFBP5 expression was detected in either cell lysate or the conditioned medium from the parental and the vector transfectant. The majority of the protein was detected in the conditioned medium from both wild type and mutant form of IGFBP5. However, the secreted wild type IGFBP5 was cleaved into a small size, in contrast, the mutant form of IGFBP5 showed mainly intact. The blot was re-probed with actin to normalize for protein loading.



3. We studied the behavior of the mutant IGFBP5 in two assays: DNA synthesis and cell migration. We found that in contrast to wild-type IGFBP5, which inhibits DNA synthesis and cell migration, mutant IGFBP5 promotes DNA synthesis and cell migration. These results support our hypothesis that localization of IGFBP5 determines its cellular function. The results are shown in figure 3 and 4.

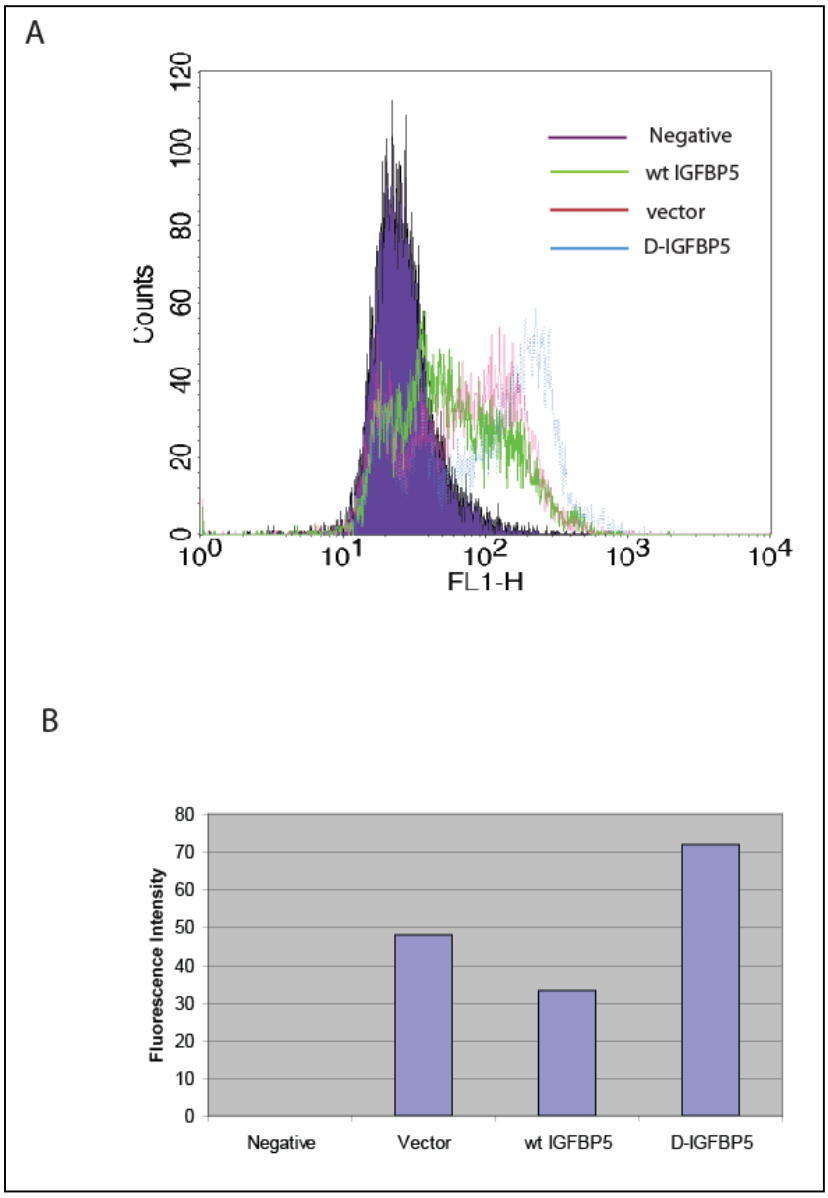
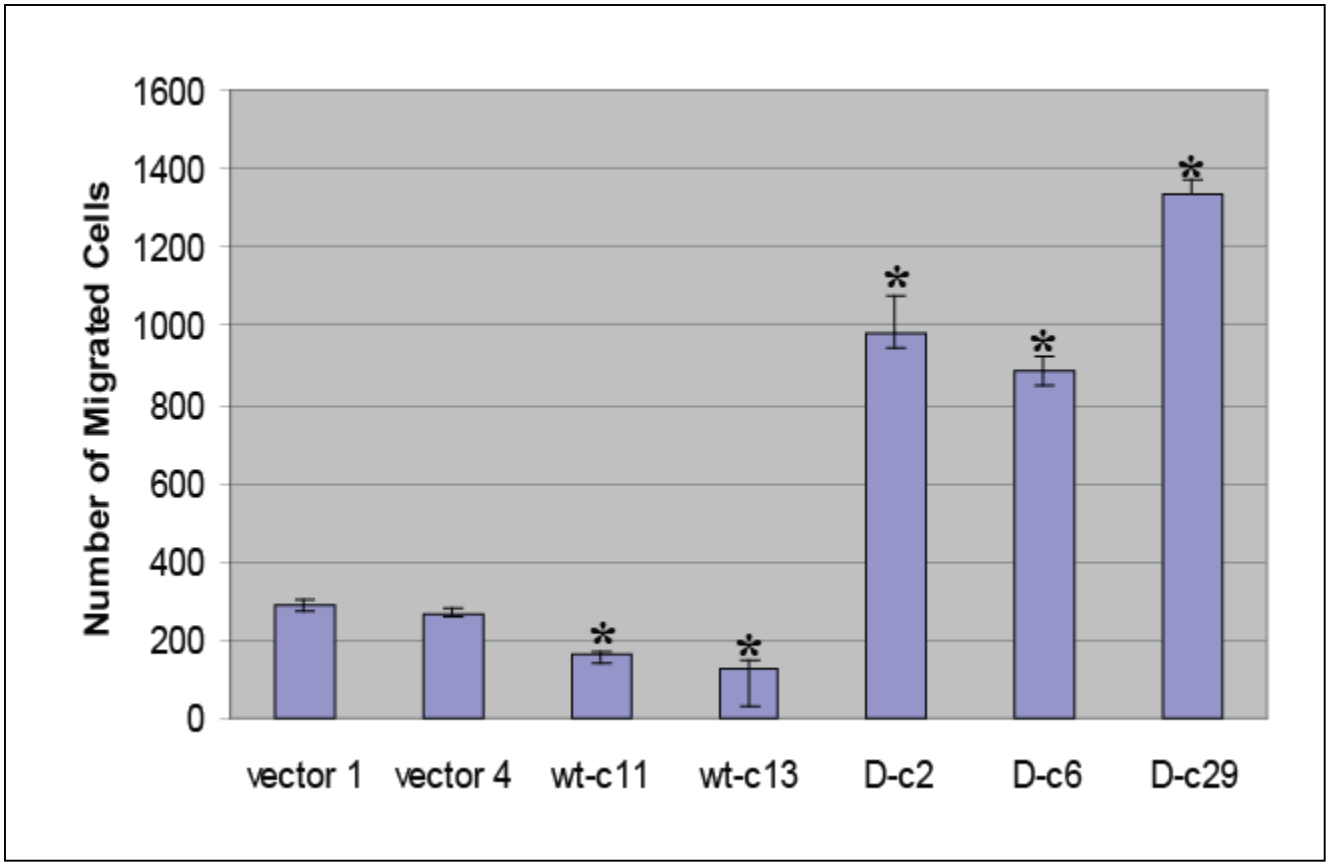


Figure 3. BrdU incorporation results. The cell growth rate for the vector and IGFBP5 transfectants was measured by BrdU incorporation using the "BrdU Flow Kits" (BD) per the manufacturer's instruction. Briefly, cells were cultured in the complete medium for 24h and then cells were incubated in the fresh complete medium with 10mM of BrdU for one hour. The incorporated BrdU was visualized by immunofluorescence staining using an anti-BrdU antibody conjugated with FITC. The geo mean fluorescence intensity from the cells was recorded by fluorescence-activity cell sorter (FACS) is shown in **A** and the same fluorescence intensity was shown as a bar graph in **B**. The cells with no anti-BrdU antibody treatment ware served as the negative control. In graph **B**, the fluorescence intensity from the negative control was graphed as zero and fluorescence intensity from the rest of samples is the value after subtracted the value from the negative control. Wild type IGFBP5 showed inhibition of cell growth compared with the vector transfectant, whereas, the mutant form of IGFBP5 (D-IGFBP5) showed promotion of cell growth.

Fig. 4 Deletion of amino acids from 214 to 218 of IGFBP5 protein promoted MBA-435 breast cancer cell motility. Quantification Data of cell migration assay. 2x10^4 cells from different cell lines as indicated were seeded into the migration chamber in serum-free Medium and 10% FBS containing medium was added in the bottom chamber. The cells were allowed to migrate for 22 to 24 hours. Then the cells were fixed and stained. All migrated cells

on the bottom side of the filter were counted. The error bars represented standard deviation from triplicate samples. The invaded cell number significantly increased from the D-IGFBP5 cell lines (*, p<0.001), whereas, The wild type IGFBP5 significantly decreased migrated cell number compared with the vector transfectant.



3. Key Research Accomplishments

We have demonstrated for the first time that the cellular localization of IGFBP5 determines whether it functions as an inhibitor of cell proliferation or promoter of cell proliferation and cell invasion. Because IGFBP5 in breast cancer patient tissues is not in the nucleus where it acts as a negative inhibitor, this study forms a foundation for future studies understanding how the cellular localization is altered in breast cancer tissues. Further, our study suggests that IGFBP5 is a target for therapy in patients. Agents that change IGFBP5 from secreted or cytoplasmic form to nuclear form are potential anti-cancer agents.

4. Reportable outcomes

This study provides evidence that non-nuclear localization of IGFBP5 in breast cancer contributes to the cancer proliferation and invasion, thus is a target for therapeutic intervention.

5. Conclusion

We will repeat the BrDu incorporation assay to ensure its reproducibility. Then we will prepare a manuscript for submission. These preliminary studies will form a basis for an application for an IDEA grant in 2007.

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